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ORIGINAL ARTICLE

Characterization of Two Species of Trypanosomatidae from the Honey Bee *Apis mellifera*: *Crithidia mellificae* Langridge and McGhee, 1967 and *Lotmaria passim* n. gen., n. sp.

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Keywords

Apidae; flagellate; Kinetoplastea; *Leptomonas*; protists; systematics; taxonomy; ultrastructure.

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ABSTRACT

Trypanosomatids are increasingly recognized as prevalent in European honey bees (*Apis mellifera*) and by default are attributed to one recognized species, *Crithidia mellificae* Langridge and McGhee, 1967. We provide reference genetic and ultrastructural data for type isolates of *C. mellificae* (ATCC 30254 and 30862) in comparison with two recent isolates from *A. mellifera* (BRL and SF). Phylogenetics unambiguously identify strains BRL/SF as a novel taxonomic unit distinct from *C. mellificae* strains 30254/30862 and assign all four strains as lineages of a novel clade within the subfamily Leishmaniinae. In vivo analyses show strains BRL/SF preferably colonize the hindgut, lining the lumen as adherent spheroids in a manner identical to previous descriptions from *C. mellificae*. Microscopy images show motile forms of *C. mellificae* are distinct from strains BRL/SF. We propose the binomial *Lotmaria passim* n. gen., n. sp. for this previously undescribed taxon. Analyses of new and previously accessioned genetic data show *C. mellificae* is still extant in bee populations, however, *L. passim* n. gen., n. sp. is currently the predominant trypanosomatid in *A. mellifera* globally. Our findings require that previous reports of *C. mellificae* be reconsidered and that subsequent trypanosomatid species designations from Hymenoptera provide genetic support.

DUE to their worldwide agricultural significance, eusocial colony behavior and semi-domestication, European honey bees (*Apis mellifera*) are model organisms for a variety of applied and pure research endeavors (Dietemann et al. 2013). Correspondingly, the suite of pathogens and symbionts they host are of heightened interest (e.g. Cox-Foster et al. 2007; Evans and Schwarz 2011; Forsgren and Fries 2010; Genersch et al. 2005; Higes et al. 2013; Klee et al. 2007; Moran et al. 2012; Vásquez et al. 2012). Currently recognized enteric unicellular parasites of *A. mellifera* represent four suprakingdom-level groups of the eukaryotes (sensu Adl et al. 2005, 2012): Amoebozoa, Chromalveolata, Excavata, and Opisthokonta. Of these, trypanosomatids belonging to Excavata (Euglenozoa: Kinetoplastea: Trypanosomatida: Trypanosomatidae) have been known to infect *A. mellifera* since at least 1912 (Fanthan and Porter 1912) with a peppering of reports in the following century supporting their generally common and global distribution including Europe (Fanthan and Porter 1912; Fyg 1954; Lom 1962; Lotmar 1946; Orantes-Bermejo 1999; Ravoet et al. 2013), Africa (Lotmar 1946), Australia (Langridge 1966; Langridge and McGhee 1967), North America (e.g. Cox-Foster et al. 2007; van Engelsdorp et al. 2009; Runckel et al. 2011), South America (Teixeira et al. 2008) and Asia (Morimoto et al. 2012; Yang et al. 2013). Reports of their taxonomic diversity have been in conflict, however, with some researchers reporting multiple species (Fanthan and Porter 1912; Fyg 1954) while others believed a single, polymorphic species was present (Langridge and McGhee 1967; Lotmar 1946). This confusion was not surprising since taxonomy of

trypanosomatids at the time relied on morphological characteristics (e.g. Hoare and Wallace 1966; Vickerman 1976; Wallace 1966), which genetic analyses have since shown are often unreliable and misleading.

Trypanosomatidae are obligate parasites and include in part genera (i.e. *Leishmania*, *Phytomonas*, *Trypanosoma*) comprised of dixenous species, requiring two hosts to complete their lifecycle (invertebrate and vertebrate or plant). Although comparatively less studied, many invertebrates are parasitized by lineages of monoxenous trypanosomatid species, requiring only one host to complete their lifecycle, and are important to consider for a clear understanding of the biology and evolutionary history of this entire family. Classically, trypanosomatids have been categorized according to six major morphotypes based on the flagellated stage of development (reviewed in Wheeler et al. 2013), yet multiple genera can be assigned to individual morphotypes. This is most pronounced with the promastigote morphotype, which has been described from the following genera: *Crithidia*, *Leishmania*, *Leptomonas*, *Herpetomonas*, *Phytomonas*, and *Wallaceina* (recently argued to be renamed as *Wallacemonas* by Kostygov et al. 2014). Thus, phylogenetics are essential for accurate taxonomic classification to circumvent homoplasies among unrelated lineages of trypanosomatids (e.g. Vickerman 1994; Votýpka et al. 2012; Wheeler et al. 2013; Yurchenko et al. 2008) and to identify cryptic species within morphologically indistinguishable populations (Schmid-Hempel and Tognazzo 2010). For this reason, current trypanosomatid research efforts rely on phylogenetics when characterizing new (e.g. Jirků et al. 2012; Maslov et al. 2010; Votýpka et al. 2013; Yurchenko et al. 2006a,b) and previously described taxa (e.g. Teixeira et al. 2011; Yurchenko et al. 2014). Nonetheless, most of these new taxa continue to be arbitrarily assigned to previously accepted genera based on morphotypes despite conflicting phylogenetic placement. This approach has artificially created polyphyletic genera within the Trypanosomatida (e.g. Merzlyak et al. 2001; Yurchenko et al. 2008) that are only now beginning to be revised based entirely on phylogenetics (Borghesan et al. 2013; Kostygov et al. 2014; Teixeira et al. 2011).

Phylogenetic analyses of trypanosomatids typically involve two nuclear DNA loci for which sequences across a large diversity of taxa are available: glycosomal glyceraldehyde-3-phosphate dehydrogenase (*gGAPDH*) and the 18S small subunit ribosomal RNA (*SSU*). These two loci have recently been used to establish the newly recognized trypanosomatid subfamily Leishmaniinae (Jirků et al. 2012), previously referred to as the slowly evolving clade (Merzlyak et al. 2001). This subfamily includes a robust lineage comprised of *Leishmania* species (the namesake of the subfamily) as well as two polyphyletic genera, *Crithidia* and *Leptomonas*. However, taxonomic research of this subfamily has focused on species derived from Hemiptera (suborder Heteroptera) or Diptera hosts, highlighting the need for additional taxa from a broader host range to clarify lineages within this subfamily (Maslov et al. 2013). The class to which trypanosomatids belong, Kinetoplastea, refers to the unique mass of kinetoplast DNA (kDNA) within the elongated mitochondria of these cells. The kDNA provides an alternative source of genetic material to which traditional nuclear phylogenetics can be compared, and its value has yet to be broadly applied by systematists. The cytochrome b gene encoded on kDNA, *Cytb*, is an important locus for distinguishing genotypes within species of *Trypanosoma* (Spotorno et al. 2008) and *Leishmania* (Asato et al. 2009) and has recently been used (in part) to characterize two closely related taxonomic lineages of trypanosomatids infecting bumblebees, *Crithidia bombi* and *Crithidia expoeki* (Schmid-Hempel and Tognazzo 2010).

Early research interests on honey bee trypanosomatids produced two key reports from *A. mellifera*. First (Lotmar 1946) was a detailed account of adult bees with a unique gut scarring pathology, termed "Schorfbienen" (scab bees), with which trypanosomatids were intimately associated and suggested to cause. Both motile, flagellated forms and nonmotile round forms (described here as "spheroids") were described as adherent to the lumenal epithelium surrounding melanized "scab" regions in a specific area of the gut called the pylorus, a highly infolded region that regulates the transition of gut contents between the ventriculus (midgut) and ileum (small intestine). Although no physical specimens were archived, detailed illustrations and photomicrographs showed these flagellates typically had acutely pointed anteriors from which the flagellum extended and enlarged rounded posteriors as well as some thin, highly elongated forms consistent with promastigote and choanomastigote morphotypes. Additional emphasis was placed on their highly specific niche within the pylorus, hence the association with melanized tissue here, which rarely extended anteriorly into the midgut or distally into the small intestine. The population was attributed to a new species with the provisional name *Leptomonas apis*, with genus assignment based on morphological standards at the time.

Two decades later another trypanosomatid from *A. mellifera* was characterized and denominated *Crithidia mellificae* (Langridge 1966; Langridge and McGhee 1967), thus becoming the first formally and widely accepted species in honey bees. While the description of *L. apis* was noted by Langridge and McGhee, their isolate was ascribed to an entirely different genus and species. This was justified by differences in cell morphology and site of tissue colonization described for each. *Crithidia mellificae* showed occasional slightly elongated promastigotes but primarily choanomastigotes, with truncated anteriors and rounded to acute posteriors. Within the host gut, *C. mellificae* primarily colonized the rectum in large numbers via spheroids attached to the lumenal surface that formed a compact, single layer of parasites. An earlier report of trypanosomatids (uncharacterized) in *A. mellifera* also recognized the rectum as a primary site of colonization (Fyg 1954). Axenic cultures of *C. mellificae* were successfully established for its characterization and thereafter, two archived type cultures from Georgia, USA were deposited by one of the authors, R. B. McGhee, to the American Type Culture Collection (ATCC): strain ATCC 30254 isolated

from *A. mellifera* and strain ATCC 30862 isolated from the wasp *Vespula squamosa* (eastern yellow jacket).

These two key descriptions provided an excellent foundation for understanding honey bee trypanosomatids. However, interest in them waned and gave way to the common practice of lumping honey bee trypanosomatids into a single taxon, with *C. mellificae* the largely accepted species. Recent applications of molecular methods used in diagnostic bee pathogen surveys (e.g. van Engelsdorp et al. 2009; Ravoet et al. 2013; Runckel et al. 2011) have confirmed the general abundance of trypanosomatids, yet the lack of clarity surrounding their taxonomy is an impediment. Studies that aimed to understand the role of microbes in diseased colonies (Cox-Foster et al. 2007; van Engelsdorp et al. 2009) used vague reference of trypanosomatid genetic signals to family (Trypanosomatidae) only, ignoring any species level insight. Alternatively, despite the historical disparity over taxa, publicly accessioned trypanosomatid genetic data have been arbitrarily assigned to *C. mellificae* when isolated from *A. mellifera* (Morimoto et al. 2012; Runckel et al. 2011, 2014; Schmid-Hempel and Tognazzo 2010) and from Asian honey bee *Apis cerana* (Yang et al. 2013), leading others to speciously designate homologous sequence data (Cornman et al. 2012; Ravoet et al. 2013). However, one study (Cornman et al. 2012) specifically used reference genetic material from a type strain of *C. mellificae* (ATCC 30254) to recognize that a divergent clade of trypanosomatids existed in honey bee colonies from the USA. This type strain of *C. mellificae* has also been used in controlled studies where they were found to stimulate complex honey bee immune responses (Schwarz and Evans 2013), identifying important implications these parasites may have on overall honey bee health.

Toward improved understanding of trypanosomatids in bees and insect trypanosomatid taxonomy, we examined the two archived type strains of *C. mellificae* (30254 and 30862) and provide the first nuclear and kDNA sequence data for this species. We also provide nuclear and kDNA sequence data from two recent trypanosomatid axenic cultures established from *A. mellifera* in the USA (strains BRL and SF) that are genetically and morphologically distinct from *C. mellificae*. Phylogenetic analyses of concatenated *gGAPDH* and *SSU* clarify three distinct clades within the Leishmaniinae subfamily: the *Leishmania* (Clade 1), the *Crithidia* (Clade 2) and a novel clade (Clade 3) that includes all trypanosomatids isolated from Hymenoptera (e.g. bees, bumblebees and wasps) and comprised of species classically assigned to either *Crithidia* or *Leptomonas* based on morphology. We propose that strains BRL and SF are type specimens (hapantotype BRL strain; parahapantotype SF strain) for a new genus and species of Trypanosomatidae within Clade 3.

MATERIALS AND METHODS

Cell line cultures

All cell lines were maintained at 25 °C axenically in "supplemented DS2" medium: Insectagro DS2 (Cellgro, Manassas, VA), 5% (v/v) fetal bovine serum (Cellgro) and 100 IU/ml penicillin—100 μg/ml streptomycin (Cellgro). Two axenic cell lines of *C. mellificae* were obtained from ATCC (Manassas, VA): (1) *C. mellificae* Langridge and McGhee (ATCC 30254) and (2) *C. mellificae* Langridge and McGhee (ATCC 30862). An axenic trypanosomatid culture isolated from the dissected ileum of an adult female *A. mellifera* at the Bee Research Lab (BRL strain ATCC PRA-422) in Beltsville, Maryland, USA was established in September 2012. The ileum was removed with sterile tools, submerged in 1 ml of supplemented DS2 medium in a 1.7-ml microtube and gently macerated with a sterile pestle. After 48 h incubation at 25 °C, an active culture was expanded in supplemented DS2 with added amphotericin B (2.5 μg/ml) until bacterial and fungal contaminants were no longer observed at which point cultures were cryopreserved. A fourth trypanosomatid axenic cell line (SF strain ATCC PRA-403) was isolated from *A. mellifera* in San Francisco, California, USA as described previously (Runckel et al. 2011).

In vivo inoculations with strain BRL

To prepare cells for inoculations, promastigotes of strain BRL were grown in supplemented DS2 media then diluted in 20% sucrose solution (1:5 sucrose to 1X phosphate buffered saline [PBS]) as described previously (Schwarz and Evans 2013). Newly emerged worker bees (< 2 d) were isolated from a colony maintained at the USDA Bee Research Lab. Bees were then divided into two treatment groups and hand fed either 5 μl of 1:1 sugar water or 5 μl of strain BRL promastigotes (10,000 cells/μl). Treatment groups were maintained separately at 34 °C + 50% relative humidity with ad libitum sterile sucrose solution. Bees were sacrificed and dissected at 10–11 d postinoculation and examined with light microscopy at 400X to 1,000X magnification.

DNA purification, gene cloning, and sequencing

DNA was extracted from cultures of axenic trypanosomatid cell lines by homogenizing cells with 1 mm glass beads in 2% (w/v) hexadecyltrimethylammonium bromide (CTAB) buffered with 100 mM Tris-HCl (pH 8.0), 1.4M NaCl, 20 mM EDTA, 0.2% 2-mercaptoethanol, 50 μg proteinase K (Promega, Madison, WI) and 5% (v/v) RNase cocktail (Life Technologies, Carlsbad, CA) using a FastPrep FP120 cell disrupter (Qbiogene, Carlsbad, CA). DNA was purified with phenol:chloroform:isoamyl alcohol (25:24:1) phase separation followed by alcohol precipitation and then resuspended in nuclease-free water.

DNA purified from each cell line was used as template for polymerase chain reaction (PCR) to clone three nuclear genome loci: (1) *gGAPDH*, (2) *SSU* rRNA, and (3) the internal transcribed spacer (*ITS*) regions from *18S* to *28S* rRNA (partial *18S*, entire *ITS-1*, *5.8S* rRNA, *ITS-2*, partial *28S* rRNA) and one mitochondrial locus, *Cytb*. Primers targeting these 4 loci were as follows: gGAPDH forward 5′-ATG GCT CCG (A/C)TC AAG GTT GGC-3′ and reverse 5′-TTA CAT CTT CGA GCT CGC G(C/G)(C/G) GTC-3′ with a 55 °C

annealing step (modified from Yurchenko et al. 2006a); SSU forward 5′-GGC GTC TTT TGA CGA ACA AC-3′ and reverse 5′-TAC GTT CTC CCC CGA ACT AC-3′ with a 60 °C annealing step (designed using Primer 3 in this study); ITS region forward 5′-GTC GTT GTT TCC GAT GAT GGT G-3′ and reverse 5′-CCT GCC AAC TTG ACA CTG C-3′ with a 57 °C annealing step (forward modified from Teixeira et al. 2008 and reverse designed in this study), Cytb forward 5′-TCG TGT AAA GCG GAG AAA GAA GA-3′ and reverse 5′-ACA CAA ACG TTC ACA ATA AAA AGC A-3′ with a 60 °C annealing step (designed in this study using Primer3).

Several honey bee samples from a Belgian trypanosomatid screening (Ravoet et al. 2013) were selected for this study. Exactly 5 μl of RNA (variable concentration) were reverse transcribed using random hexamer primers with the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Vilnius, Lithuania). Four specimens of *Osmia bicornis* were also collected in May 2012 at campus Sterre, Ghent, Belgium. Temporary trypanosomatid cultures (with contaminating bacteria) were established using a described protocol (Popp and Latorff 2011), from which DNA was extracted with DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) following the protocol for cell cultures. To amplify trypanosomatid specific genes from infected bees (*A. mellifera*, *O. bicornis*), previously published primers were used for *gGAPDH* (Yang et al. 2013), *Cytb* was amplified using the primer Tryp-cytb-F 5′-TGT GGW GTK TGT TTA GC-3′ and Tryp-cytb-R 5′-CRT CWG AAC TCA TAA AAT AAT G-3′ with a 50 °C annealing step, and *SSU* was amplified using the above primers designed for this study. PCR reactions contained 2 μM of forward and reverse primer; 1 mM $MgCl_2$; 1.25 U Hotstar HiFidelity DNA polymerase (Qiagen) and 1 μl cDNA (*A. mellifera*) or 100 ng DNA (*O. bicornis*).

Column purified (QIAprep®; Qiagen) recombinant plasmid amplicons were bidirectionally sequenced with T7 and SP6 priming sites using BigDye® Terminator on a ABI3730XL capillary sequencer (Macrogen, Rockville, MD). Sequencher 5.2.3 (Gene Codes Corporation, Ann Arbor, MI) software was used to assess sequence quality, unambiguously determine DNA sequence, and build contigs for each insert. All nonredundant sequences isolated were accessioned to GenBank and are presented in Tables S1, S3–S6.

Phylogenetics

Previously, accessioned trypanosomatid sequences were retrieved from GenBank and aligned with sequences we recovered from each locus using MUSCLE (Edgar 2004) with minor hand correction to minimize gaps. Single locus alignments were analyzed using both maximum likelihood (ML) discrete-character method and neighbor-joining (NJ) distance method in MEGA 6.06 for MacOS (Tamura et al. 2013). Best-fit nucleotide substitution testing of 24 models based on lowest Bayesian information criterion (BIC) inferred by nearest neighbor interchange heuristic search determined the General Time Reversible (GTR) + discrete Gamma Distribution (G) + evolutionarily invariable sites (I) was optimal for ML analysis of *gGAPDH* and *SSU* while Tamura-Nei (TN93) +G was optimal for *Cytb*. All NJ method analyses used Tamura–Nei model including transitions and transversions, uniform rates among sites and pairwise deletion of gaps/missing data. All sites in aligned regions were used and areas of no coverage in shorter sequences were ignored. Since both *gGAPDH* and *SSU* alignments had BIC support for the same optimal model, we generated a concatenated alignment for analysis using the most commonly recovered sequence variants from each locus to create a single, representative sequence for each trypanosomatid cell line. Tree topologies were tested with 1,000 bootstraps using *Bodo saltans* (*SSU* and *gGAPDH*) or *Trypanosoma cruzi* and *Trypanosoma brucei* (*Cytb*) species as outgroups. Optimal phylograms were imported to TreeGraph 2 (Stöver and Müller 2010) for editing. The trees and associated sequence alignments shown in this manuscript (Fig. 1, 2) are available in TreeBASE ID 16436 at http://purl.org/phylo/treebase/phylows/study/TB2:S16436 or upon request.

Confocal laser scanning microscopy

Live cells were pelleted from axenic cultures for 5 min at 425 *g*, fixed in 4% paraformaldehyde in 1X PBS (w/v) for 30 min at 4 °C, pelleted at 239 *g* and resuspended in 1X PBS then stained using 4′,6-diamidino-2-phenylindole (DAPI) (NucBlue® Fixed Cell Stain; Life Technologies) for 5 min. Stained cells were viewed with differential interference contrast (DIC) on a Zeiss™ LSM710 confocal laser scanning microscopy (CLSM) system using a Zeiss Axio Observer™ inverted microscope as described previously (Macarisin et al. 2010, 2012).

Electron microscopy

Scanning electron microscopy

Live cells were pelleted for 5 min at 425 *g* and fixed with 3% glutaraldehyde in 0.05M PBS for 2 h at room temperature then overnight at 4 °C. Fixed cells were washed six times in PBS then dehydrated in a graded series of ethanol. Samples were critical point dried in a Tousimis Samdri-780A (Tousimis Research Corporation, Rockville, MD), placed onto ultra smooth (12 mm diam.) carbon adhesive discs (Electron Microscopy Sciences, Inc., Hatfield, PA) attached to 15 cm × 30 cm copper plates and sputter coated using a magnetron sputter head equipped with a platinum target. Samples were observed in an S-4700 field emission SEM (Hitachi High Technologies America, Inc., Dallas, TX) equipped with a Quorum CryoPrep PP2000 (Quorum Technologies Ltd, East Sussex, UK) cryotransfer system using accelerating voltage of 5 kV. Images were captured using a 4pi Analysis System (Agilent Technologies, Durham, NC).

Transmission electron microscopy

Live cells were fixed in 2.5% glutaraldehyde (v/v) in 0.1M sodium cacodylate buffer (pH 7.4) for 2 h at room

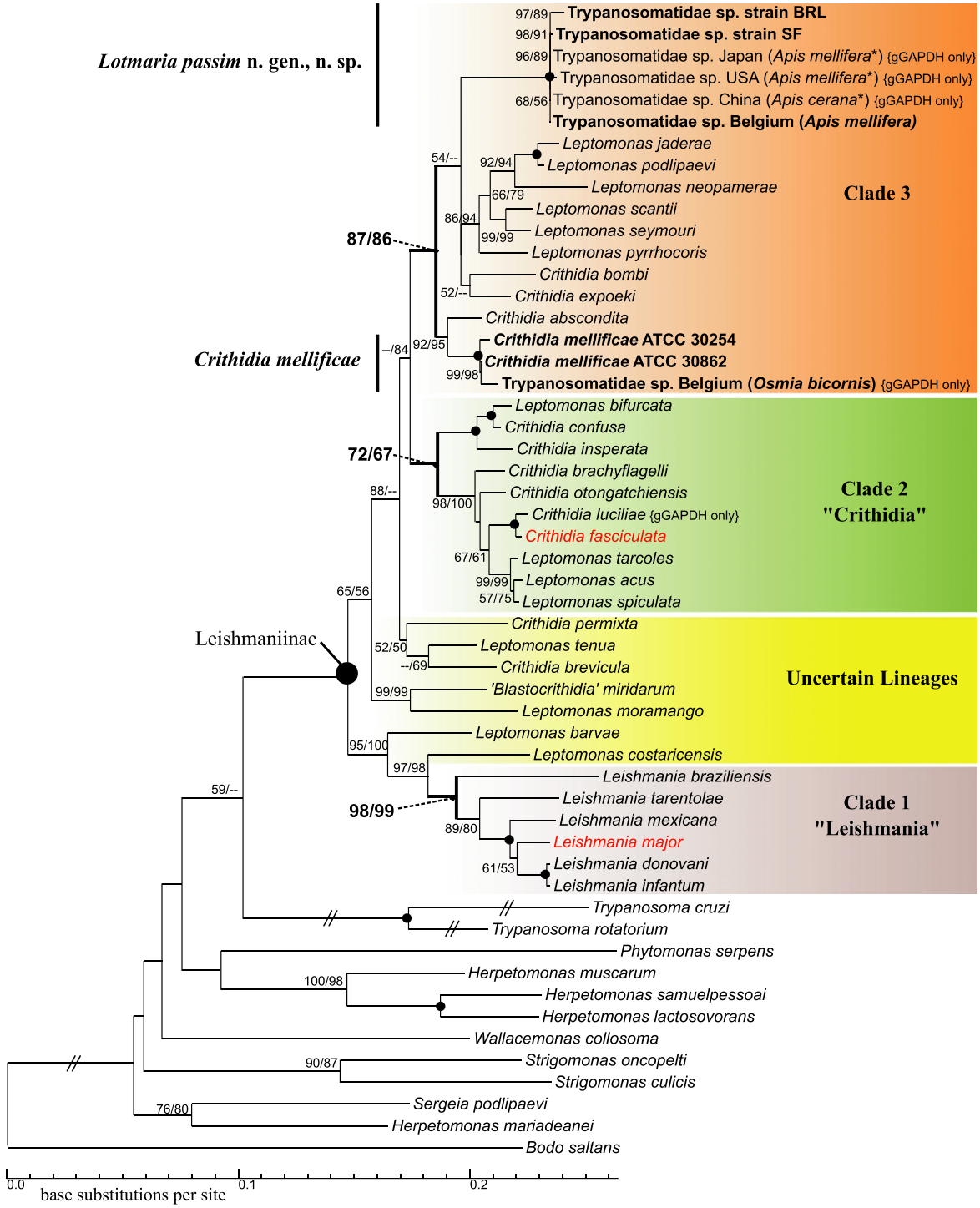

Figure 1 Phylogeny reconstruction by ML method using concatenated *gGAPDH* and *SSU* sequences from four axenic strains (30254/30862 *Crithidia mellificae*; BRL/SF *Lotmaria passim* n. gen., n. sp.) and from in vivo isolated sequences. Alignments were made with previously accessioned Kinetoplastea (accession numbers available in TreeBASE ID 16436), totaling 53 sequences and 1,978 sites. Both loci were independently best modeled using GTR+G+I based on lowest BIC, justifying concatenation and analysis with the same model. Original sequences are in bold font, represented by the most prevalent sequence variants from each strain. Node support values for ML and NJ methods are shown, respectively, with support < 50% (1,000 bootstraps) not shown and 100% support using both methods indicated with a black circle. Some taxa for which only the *gGAPDH* locus was available are noted. The base of the Leishmaniinae subfamily is indicated and robust clades within are highlighted (Clade 1, 2, and 3). Country of origin and host species are given for noncultured isolates. *Originally accessioned as *C. mellificae*. Cross-hatched branches are half their original length.

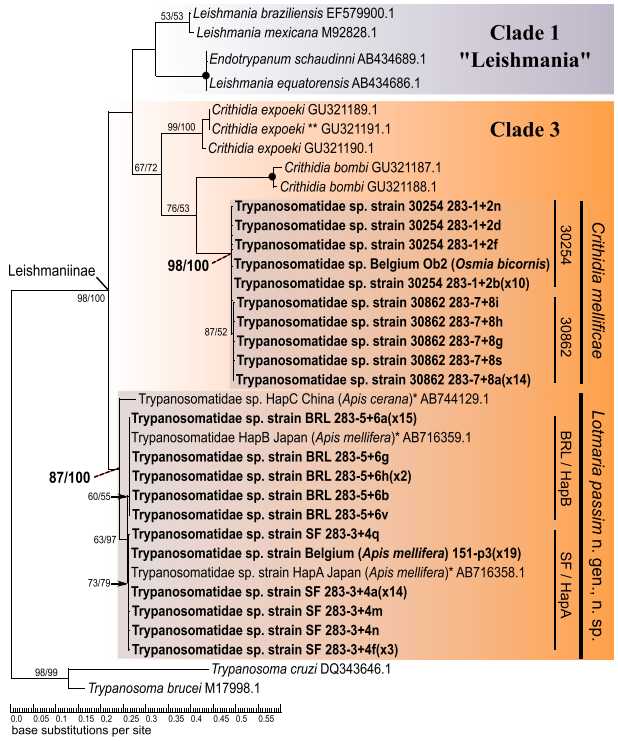

Figure 2 Trypanosomatid phylogeny reconstruction using *Cytb* sequences provides resolution of genotype lineages within Clade 3. Sequence alignments (593 sites) were analyzed by ML and NJ methods and best-fit model TN93 + G ($G = 0.2521$; $\ln L = -3{,}726.7857$ from lowest BIC = 8,728.709), with branch support > 50% (1,000 bootstraps) shown, respectively. Nodes with black circles have 100% support. All original sequences are shown in bold font with the multiple of identical sequences recovered in parentheses where applicable. Country of origin and host species are given for noncultured isolates. For brevity, select leaves are shown from the original tree (Fig. S7, S8). *These sequences were originally accessioned as *Crithidia mellificae*. **Accessioned as *C. mellificae* (Schmid-Hempel and Tognazzo 2010) but identified here as *Crithidia expoeki* based on sequence identity to this species.

temperature then overnight at 4 °C. Samples were then rinsed six times in 0.1M sodium cacodylate buffer, postfixed in 2% (w/v) osmium tetroxide for 2 h, dehydrated in ethanol series then embedded in acrylic resin (LR White Resin System, London, UK) and cured at 55 °C for 24 h prior to sectioning using a Reichert-Jung/AO ultra-microtome fitted with a Diatome diamond knife. Sections were stained with 4% uranyl acetate and 3% lead citrate, viewed in an HT-7700 microscope (Hitachi High Technologies America) at 80 kV and imaged using an AMT High-Res CCD camera.

RESULTS

Genetic characterization of four axenic trypanosomatid strains

DNA templates from each of the *C. mellificae* type strains (30254 and 30862) as well as two recent axenic isolates from *A. mellifera*, strain BRL and strain SF, were PCR amplified at three nuclear loci (*gGAPDH*, *SSU*, and *ITS1-5.8S-ITS2*) and one kDNA (*Cytb*) locus. From 226 total recombinant plasmid clone inserts, we obtained 99 unique sequences in total that were accessioned to GenBank as reference material for these four strains (Table S1).

Nucleotide sequence identity among the axenic strains at these four loci revealed two consistent and distinct taxonomic groups: strains BRL and SF each shared higher identity with one another than with strains 30254 or 30862 (Table 1). Nucleotide identity among sequences from strains BRL and SF ranged from 98.09% (*ITS1-5.8S-ITS2*) to 100% (*SSU*) while identity from strains 30254 and 30862 ranged from 97.12% (*gGAPDH*) to 100% (*SSU*). When sequences from strains BRL and SF were both compared to those of strains 30254 and 30862, identity ranged from 64.69% (*ITS1-5.8S-ITS2*) to 95.48% (*SSU*) between these two taxonomic groups. Sequence identity between taxonomic group BRL/SF compared to group 30254/30862 was consistently lower at all four loci than sequence identity among strains BRL/SF or among 30254/30862.

Variation among sequences at the *gGAPDH* and *Cytb* locus were due to single nucleotide polymorphisms (SNPs) while the *SSU* and *ITS1-5.8S-ITS2* loci included variation due to SNPs and insertions/deletions (indels). Homologous regions of *SSU* were 10 bp shorter in strains 30254 and 30862 (763 bp) compared to strains BRL and SF (773 bp). Cloned fragments spanning the full *ITS1-5.8S-ITS2* region in strains BRL and SF ranged from 1,492 to 1,511 bp (1,519 aligned sites) and ranged from 1,279 to 1,294 bp (1,296 aligned sites) in strains 30254 and 30862.

To assess potential functional variation, we contrasted predicted protein sequences of gGAPDH and Cytb (Table S2). The range of identities at both loci formed two, non-overlapping groups: one was comprised of strains 30254/30862 and the other of strains BRL/SF. Identity across 347 predicted amino acids of gGAPDH ranged from 97.6% to 100% within strains BRL/SF and 97.9% to 100% within strains 30254/30862. Between these two taxonomic groups, identity ranged from 92.7% to 95.9%. Similarly, the 195 predicted amino acids of Cytb showed strains BRL/SF were more similar to each other than to any 30254/30862 sequences and vice versa (both were 99.4–100% identical within group). Contrasting the two groups to each other, identity ranged from 97.9% to 98.4%.

Trypanosomatid sequences isolated in vivo from bees

Fragments of trypanosomatid nucleic acids (*gGAPDH*, 492 bp; *SSU*, 763–775 bp; *Cytb*, 452 bp) amplified directly out of *A. mellifera* and *O. bicornis* (red mason bee) collected in Belgium recovered 39 (*A. mellifera*) and 2 (*O. bicornis*) unique sequences that shared high sequence identity to either taxonomic group BRL/SF or 30254/30862. Nearly all sequences isolated from *A. mellifera* (*gGAPDH*, KM066212–KM066224; *SSU*, KM066227–KM066239; *Cytb*, KM066240–KM066250) had high identity

Table 1. Inter- and intraspecific identity among unique DNA sequences from *Lotmaria passim* n. gen., n. sp. (strains BRL and SF) and *Crithidia mellificae* (strains 30254 and 30862) trypanosomatid isolates at four genetic loci

	Cytb	gGAPDH	SSU	ITS1-5.8S-ITS2
Identity (%) between *L. passim* and *C. mellificae*				
BRL vs. 30254	88.4–88.62	92.32–92.80	94.84–95.48	64.69–65.69
SF vs. 30254	88.29–88.46	93.18–93.66	94.84–95.48	65.57–66.50
BRL vs. 30862	88.12–88.29	91.07–92.89	95.35–95.48	65.57–65.69
SF vs. 30862	87.95–88.12	91.93–93.76	95.23–95.48	66.44–66.50
Alignment length	589 sites	1,041 sites	775 sites	1,606 sites
Identity (%) within *L. passim*				
BRL vs. SF	99.32–99.49	98.66–99.04	99.74–100	98.35–99.14
BRL only	99.66–99.83	99.62–99.71	99.87	98.48–99.87
SF only	99.66–99.83	98.85–99.81	99.74–99.87	98.09–99.93
Alignment length	589 sites	1,041 sites	773 sites	1,519 sites
Identity (%) within *C. mellificae*				
30254 vs. 30862	99.49–99.66	97.12–99.23	99.87–100	98.46–98.77
30254 only	99.66–99.83	98.94–99.42	99.34–99.87	98.53–99.38
30862 only	99.66–99.83	97.69–99.90	99.87	99.54–99.92
Alignment length	589 sites	1,041 sites	763 sites	1,296 sites

to sequences of group BRL/SF (99.34–100%). However, two *SSU* sequences recovered from *A. mellifera* (KM066225, KM066226) were 99.87% and 100% identical to taxonomic group 30254/30862. The two trypanosomatid sequences recovered from *O. bicornis* also belonged to the 30254/30862 group (*gGAPDH*, KM066211, 99.56%; *Cytb*, KM066251, 100%).

Phylogenetic analyses

Unambiguous and unique *gGAPDH*, *SSU* and *Cytb* DNA sequences isolated from axenic strains 30254, 30862, BRL and SF along with sequences isolated in vivo from infected honey bees (*A. mellifera*) and red mason bees (*O. bicornis*) were aligned to homologous sequences extracted from GenBank that represented a broad range of available Trypanosomatidae taxa. We produced *gGAPDH*, *SSU*, and *Cytb* alignments containing 160, 126, and 51 sequences in total, respectively. Concatenated *gGAPDH-SSU* analyses (Fig. 1) placed all of our Trypanosomatidae strains (30254, 30862, BRL, SF) into a novel clade within the Leishmaniinae subfamily (sensu stricto Maslov and Lukeš in Jirků et al. 2012) adjacent to two additional clades with strong ML and NJ bootstrap support, respectively: Clade 1 (*Leishmania*; 98, 99), Clade 2 (*Crithidia*; 72, 67) and novel Clade 3 (87, 86). Within Clade 3, our sequences consistently resolved into two clearly distinct taxonomic units with strong support (bootstrap % for ML and NJ methods): strains 30254/30862 (99, 98) and strains BRL/SF (100, 100). Single gene trees for *gGAPDH*, *SSU*, and *Cytb* robustly resolved all sequence variants according to these two taxonomic groups as well, among a broader range of previously accessioned Trypanosomatidae sequences (Fig. S1–S8). First, all sequences isolated from strains 30254 and 30862 (*C. mellificae*) in addition to two *SSU* sequences isolated in vivo from *A. mellifera* (Fig. S2) and two sequences isolated in vivo from *O. bicornis, gGAPDH* (Fig. S1) and *Cytb* (Fig. 2) formed a novel taxonomic unit (ML and NJ bootstrap support %, respectively): *gGAPDH* (98, 100), *SSU* (93, 99), *Cytb* (98, 100). A second clade consistently resolved was comprised of all sequence variants from strains BRL and SF as well as all but two sequences isolated in vivo from *A. mellifera* in Belgium (ML and NJ bootstrap support %, respectively): *gGAPDH* (98, 100), *SSU* (99, 96), *Cytb* (87, 100).

All trypanosomatid sequences previously accessioned as *C. mellificae* belong to the BRL/SF clade and not to the 30254/30862 *C. mellificae* clade (Fig. 1, 2, S1–S8). *gGAPDH* sequences isolated from *A. mellifera* in the USA (JF423199) and Japan (AB716357) and a *gGAPDH* sequence isolated from *Apis cerana* (Asian honey bee) in China (AB745489) are all members of the BRL/SF clade (Fig. 1, indicated by *). An *SSU* sequence (GU321196) isolated from *A. mellifera* in Switzerland (Fig. S2, indicated by *) and three *Cytb* sequences (AB716358, AB716359 and AB744129) originally ascribed to *C. mellificae* as "haplotype" variants A, B, and C, respectively (Fig. 2, indicated by *), all belong to the BRL/SF clade as well. BLAST heuristic algorithm searches using promiscuous to strict parameters did not identify additional sequences belonging to the BRL/SF or 30254/30862 clades not already included in our alignments.

Despite flawed species designations, "haplotype" distinctions made previously using *Cytb* (Morimoto et al. 2012) are corroborated by our data and show strain BRL forms a distinct subclade (ML = 60, NJ = 55) with "haplotype B" sequence and strain SF forms a distinct subclade (ML = 73, NJ = 79) with "haplotype A" sequence (Fig. 2, S7, S8). None of the sequences we isolated were within the "haplotype C" subclade, represented solely by a sequence from *A. cerana*, yet our analyses support that this sequence is a lineage of the BRL/SF clade. Similarly, distinct subclades within the 30254/30862 clade were resolved using *Cytb*, such that the five unique sequences obtained from strain 30862 formed a distinct subclade from strain 30254 by ML (87) and NJ (52) analyses. The

Cytb sequence we isolated from *O. bicornis* clustered with the 30254 subclade. Finally, one *Cytb* sequence (GU321191) originally accessioned as *C. mellificae* clearly belongs to the *C. expoeki* clade (Fig. 2, indicated by **). Tables S3–S6 correlate sequence names used in the trees to their GenBank accession numbers for novel sequences obtained from this study.

Morphology and ultrastructure

Cultures of *C. mellificae* 30254 and 30862 had noticeably different predominant cell morphology when compared to those of strain BRL and SF. Representative confocal microscopy images show the predominant morphology of *C. mellificae* 30254 (Fig. 3A) and strain BRL (Fig. 3D) in culture, both of which had a single, long, free flagellum inserted at the apical end of the cell. We regularly observed cells with the choanomastigote morphology in cultures of both *C. mellificae* isolates (30254 and 30862) and cell polymorphism consistent with what has already been described (Langridge and McGhee 1967). In contrast, BRL and SF strains predominantly exhibited more elongated, tear-drop shaped cells typical of a promastigote morphotype that narrowed posteriorly to a short caudate (tail-like) extension. Brightly fluorescent kDNA and slightly more diffusely fluorescent nuclear DNA were localized with DAPI fluorescence and CLSM. The kDNA was located anterior (closer to the flagellum insertion point) to the nucleus in both *C. mellificae* 30254 (Fig. 3B, C) and strain BRL (Fig. 3E, F) cells.

Measurements from microscopy images of *C. mellificae* 30254 choanomastigotes showed cells ($n = 50$) averaged 6.62 μm in length (SD ± 1.23 μm, range 4.61–8.88 μm) by 3.32 μm widest width (SD ± 0.43 μm, range 2.47–4.38 μm). Average promastigote length of strain BRL cells ($n = 50$) were 7.44 μm in length (SD ± 1.59 μm, range 4.66–11.40 μm) by 3.15 μm widest width (SD ± 0.76 μm, range 1.50–4.65 μm). These measurements were made on the dominant morphotype from axenic cultures and do not include transitional variants that were approaching or at the spheroid stage.

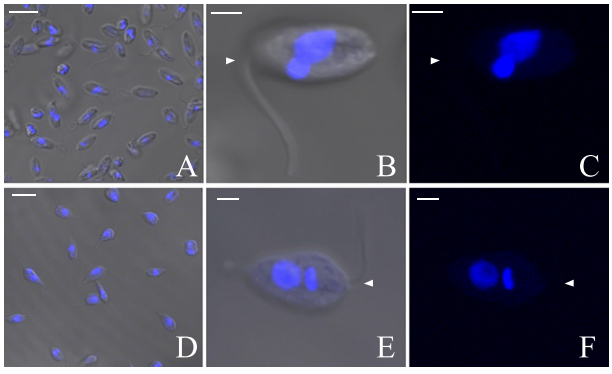

Figure 3 Confocal microscopy of *Crithidia mellificae* strain 30254 (**A–C**) and *Lotmaria passim* n. gen., n. sp. strain BRL (**D–F**) showing the smaller and overall brighter kinetoplast in relation to the larger and less bright nucleus. Typical cell morphology of fixed axenic cell culture with DAPI fluorescence and DIC (A, D). Single cell view of fixed and DAPI-stained *C. mellificae* with DIC and fluorescence (B) and with fluorescence only (C). DAPI-stained *L. passim* with fluorescence and DIC (E) and with fluorescence only (F). Arrows point to the anterior flagellum insertion point. Scale bars: 10 μm (A, D) and 2 μm (B, C, E, F).

Scanning electron microscopy (Fig. 4A–C) and transmission electron microscopy (TEM) (Fig. 4D–K) imaging of *C. mellificae* 30254 revealed the choanomastigotes had deep, narrow lateral grooves (Fig. 4A–C) formed by the plasma membrane that were most likely structurally supported by a network of subpellicular microtubules (Fig. 4D, K). A single flagellum inserted into a narrow, anterior pocket that extended into approximately half the length of the cell (Fig. 4D, H) with no apparent spicules or extensions at the point of insertion (Fig. 4B arrowhead). The flagellum was comprised of an axoneme (Fig. 4D, E) with typical 9 × 2 + 2 microtubule structure (Fig. 4F) and a cryptic paraflagellar rod (PFR; Fig. 4H–J). kDNA was adjacent to the basal bodies at the base of the flagellum (Fig. 4E) within an elongated mitochondrion (Fig. 4E–G). TEM imaging corroborated confocal microscopy results that showed kDNA lies just anterior to the adjacent nucleus (Fig. 4D, I), which showed an electron dense nucleolus (Fig. 4G). Additional typical trypanosomatid cell structures included glycosomes, acidocalcisomes and spongiome (Fig. 4D).

The unique promastigote cell morphology of strain BRL was more clearly discerned via SEM (Fig. 5A–C, E) and TEM (Fig. 5D, F–L) from that of *C. mellificae* 30254. In addition to the caudate, posterior extension (Fig. 5A–C, K) BRL promastigotes had a broad, deep lateral groove (Fig. 5A–C) in contrast to the narrow grooves of *C. mellificae* (Fig. 4A–C). Distinguishing these strains further was the common presence of a short spicule that extended from the flagellar pocket (Fig. 5C, D arrowheads) of strain BRL promastigotes that appeared to arise from the flagellum at its insertion point into the flagellar pocket, and thus did not appear to be emergent flagella from early cell division since each flagellum originated deep within the flagellar pocket and was comprised of an axoneme independently surrounded by plasma membrane (Fig. 5I arrowheads). Furthermore, flagellated cells from both *C. mellificae* 30254 (Fig. 4I) and BRL (Fig. 5E, F) cultures were regularly seen undergoing cytokinesis yet only BRL strain cells were observed to have these spicules. The flagellar pocket of strain BRL did not extend as deeply into the cell (Fig. 5K) as *C. mellificae*. kDNA within a large extended mitochondrion lying just beneath the subpellicular microtubule network (Fig. 5H) was localized anterior and adjacent to the nucleus (Fig. 5K, L), as determined with DAPI staining (Fig. 3D–F), at the base of a flagellum that had an axoneme with 9 × 2 + 2 microtubule structure and a proximal cryptic PFR (Fig. 5G, J). Additional ultrastructural features included a large nucleus and nucleolus, acidocalcisomes, glycosomes, basal bodies, and a contractile vacuole (Fig. 5D, F, J–L).

In addition to typical cytokinesis, two additional states of intercellular adherence were regularly observed in our

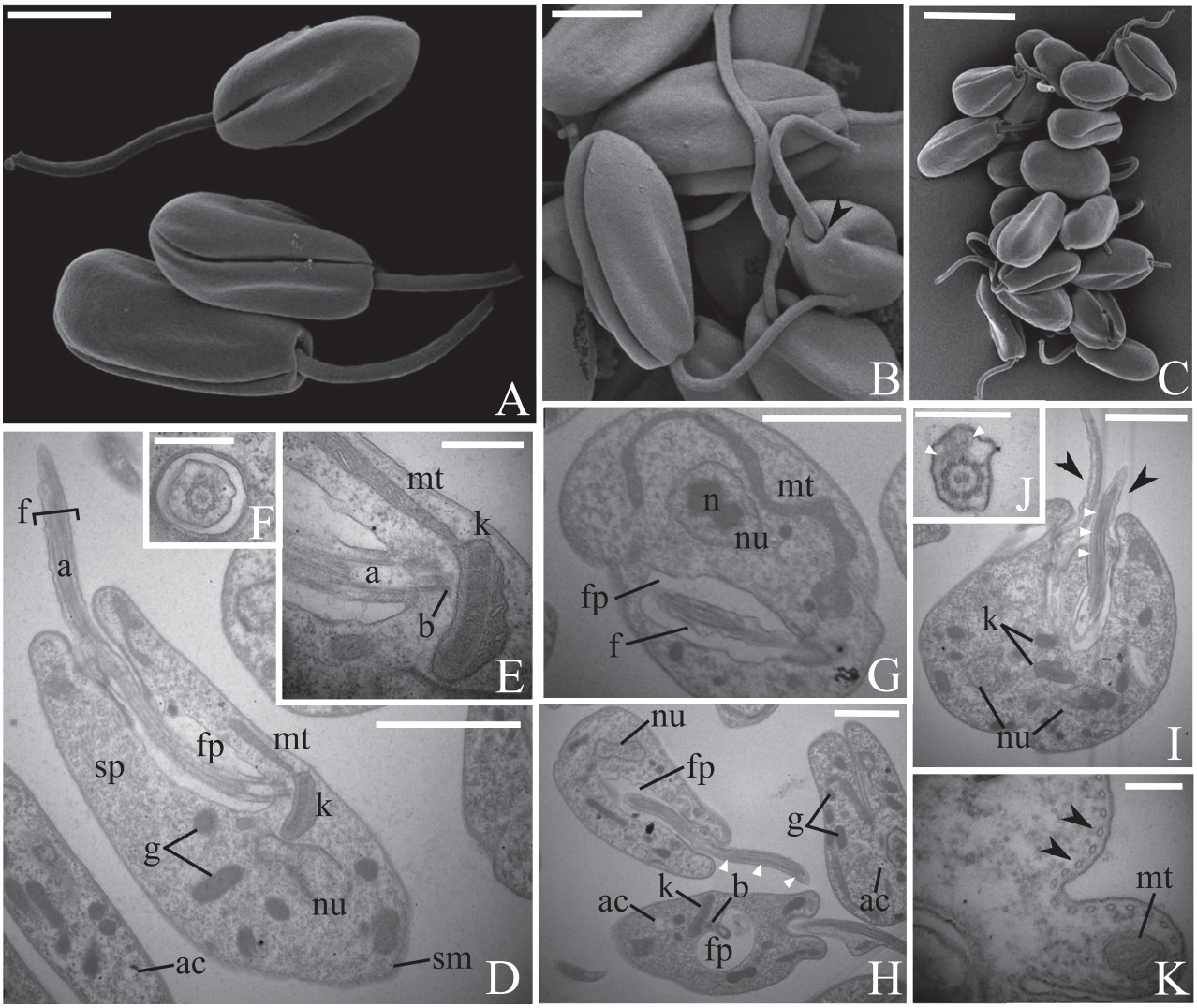

Figure 4 SEM (**A**–**C**) and TEM (**D**–**K**) images of *Crithidia mellificae* strain 30254. **A**–**C**. Views of typical choanomastigotes with multiple deep, narrow lateral grooves and anterior flagellum insertion point. Anterior view into insertion point of flagellum (B, arrowhead). **D, H.** Longitudinal sections of choanomastigotes showing typical cell and organelle features. The paraflagellar rod (PFR) is indicated (H, white arrowheads). **E**. Higher magnification of (D) showing kinetoplast in extended mitochondrion, microtubules of the axoneme and basal bodies. **F.** Cross-section of flagellar pocket (seen as the plasma membrane ring encircling the flagellum) showing 9 × 2 + 2 microtubule architecture of the axoneme. **G**. Cross-section of a choanomastigote showing extended mitochondrion. **I**. Cytokinesis in progress along the longitudinal axis with two flagella (black arrowheads), kinetoplasts and nuclei visible. PFR is indicated (white arrowheads). **J.** Cross-section of a free flagellum with PFR (white arrowheads). **K**. Subpellicular microtubule network (black arrowheads) visible beneath the plasma membrane shown in cross-section at a lateral groove. Abbreviations: acidocalcisome ("ac"), axoneme ("a"), basal bodies ("b"), flagellum ("f"), flagellar pocket ("fp"), glycosome ("g"), kinetoplast ("k"), mitochondrion ("mt"), nucleus ("nu"), nucleolus ("n"), subpellicular microtubules ("sm"), spongiome ("sp"). Scale bars: 5 μm (C), 2 μm (A, B, D, G–I), 500 nm (E, F, J), 200 nm (K).

trypanosomatid cell cultures (not shown). First, both *C. mellificae* 30254/30862 and BRL/SF strain cells would occasionally form aggregates of up to ~30 cells by entwining their flagella, forming what are known as "rosettes" (Dwyer et al. 1974). Second, adherent pairs of BRL/SF strain promastigotes occasionally formed when two cells conjoined via the caudate extensions, in a posterior–posterior fashion, forming a "doublet" (Wheeler et al. 2011). This form of intercellular adherence was not observed in *C. mellificae* cultures. Both of these examples of intercellular adherence may have been artifacts of cell culture since they were not observed in vivo.

Tissue tropism of strain BRL

Primary site of colonization in experimentally infected bees (n = 20) was the anterior rectum, particularly surrounding the rectal papillae, often extending into the distal end of

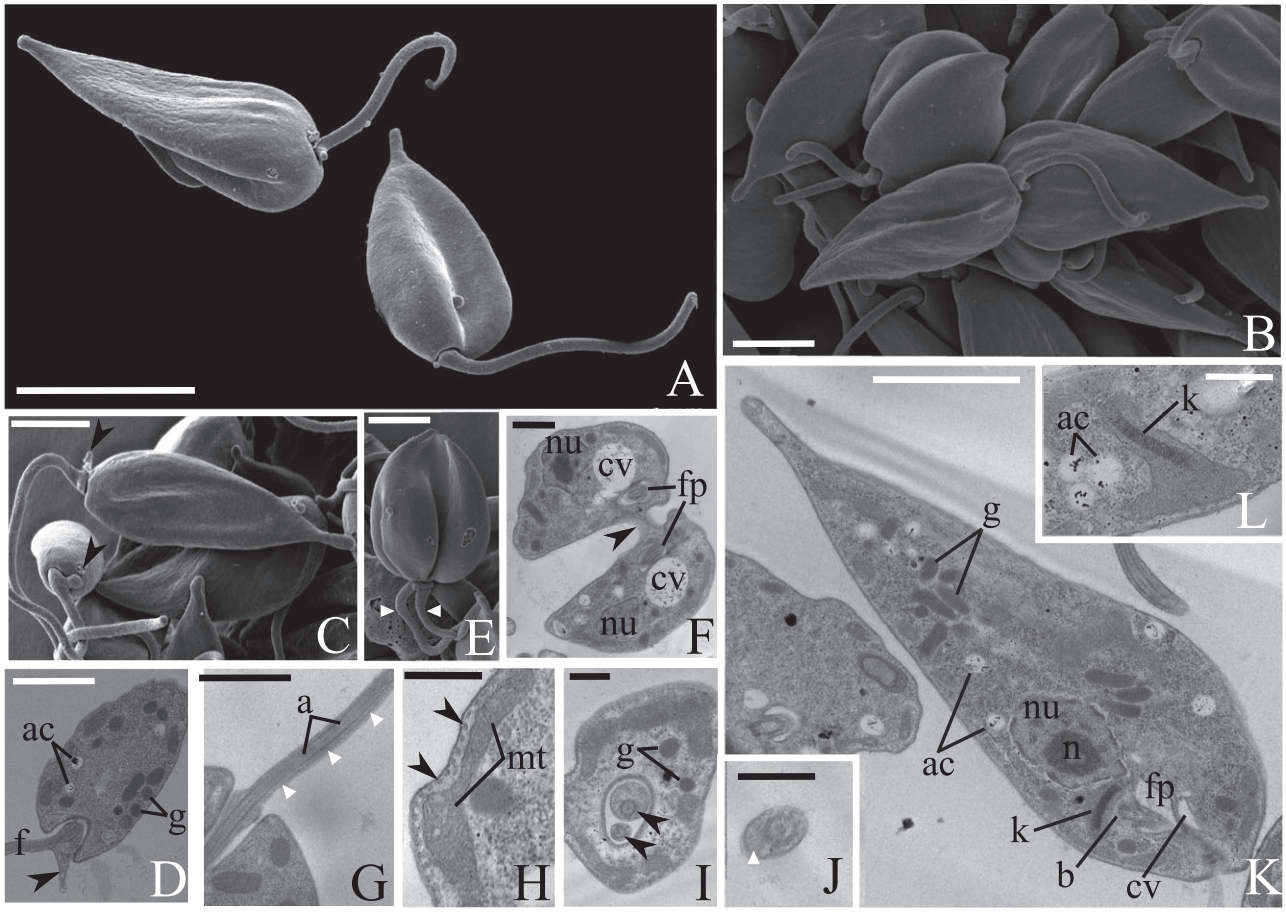

Figure 5 SEM (**A**–**C**, **E**) and TEM (**D**, **F**–**L**) of *Lotmaria passim* n. gen., n. sp. strain BRL. **A, B**. Typical lanceolate promastigote cell morphology with broad, deep lateral groove, anterior flagellum insertion point and caudate posterior extension visible. **C, D**. A commonly present spicule (black arrowheads) at the insertion point of the flagellum is an extension of the plasma membrane from the flagellum (D). **E, F**. Cytokinesis along the longitudinal axis seen using SEM (E) with two developed flagella (white arrowheads) and in TEM cross-section (F) where only a small section of plasma membrane (black arrowhead) remains holding the two cells together. **G.** Longitudinal section of flagellum proximal to the cell body with paraflagellar rod (PFR, white arrowheads) alongside the axoneme microtubules. **H, I.** Cross-sections of promastigotes showing subpellicular microtubule network (H, black arrowheads) beneath the plasma membrane and early stage cell division (I) showing two flagella developed within the flagellar pocket (black arrowheads). **J**. Cross-section of free flagellum showing cryptic PFR (white arrowhead). **K, L.** Longitudinal sections of promastigotes showing typical cell and organelle ultrastructure. A portion of the extending mitochondrion from the kinetoplast is shown in detail (L). Abbreviations: acidocalcisome ("ac"), axoneme ("a"), basal bodies ("b"), contractile vacuole ("cv"), flagellum ("f"), flagellar pocket ("fp"), glycosome ("g"), kinetoplast ("k"), mitochondrion ("mt"), nucleus ("nu"), nucleolus ("n"). Scale bars: 4 μm (A), 2 μm (B–E, K), 1 μm (F, G), 500 nm (H–J, L).

the ileum just proximal to the rectum. The lumenal surfaces of these sites in uninfected bees (Fig. 6A) were noticeably different from infected bees, which were largely covered by a dense layer of spheroids (Fig. 6B, C) approximately 3–4 μm in diam. When disrupted from their in vivo location (Fig. 6D–I), these cells often separated from one another. Spheroids were only observed in the hindgut and were common in the rectum ($n = 17$) and occasional in the ileum ($n = 8$). Within the ileum, spheroids were almost exclusively observed attached at the distal end proximal to the rectum, except for one observation in the pylorus of the ileum ($n = 1$). No spheroids were observed in the crop or midgut.

Less numerous but consistently present were promastigotes distributed among the spheroids on the hindgut lumenal surface (Fig. 6C, D). In vivo morphology of promastigotes was identical to those from axenic culture and showed typical acute posteriors (Fig. 6G, H) and a deep lateral groove (Fig. 6I). Rarely, more rounded (blunt posterior) promastigotes were observed in the ileum and rectum (not shown). Promastigotes also displayed the spiraling motility as observed from axenic cultures. Promastigotes were common in the rectum ($n = 17$), occasional in the ileum ($n = 10$), and rare in the midgut ($n = 1$) and crop ($n = 2$). Three bees from the experimentally infected group did not have detectable trypanosomatids at the time

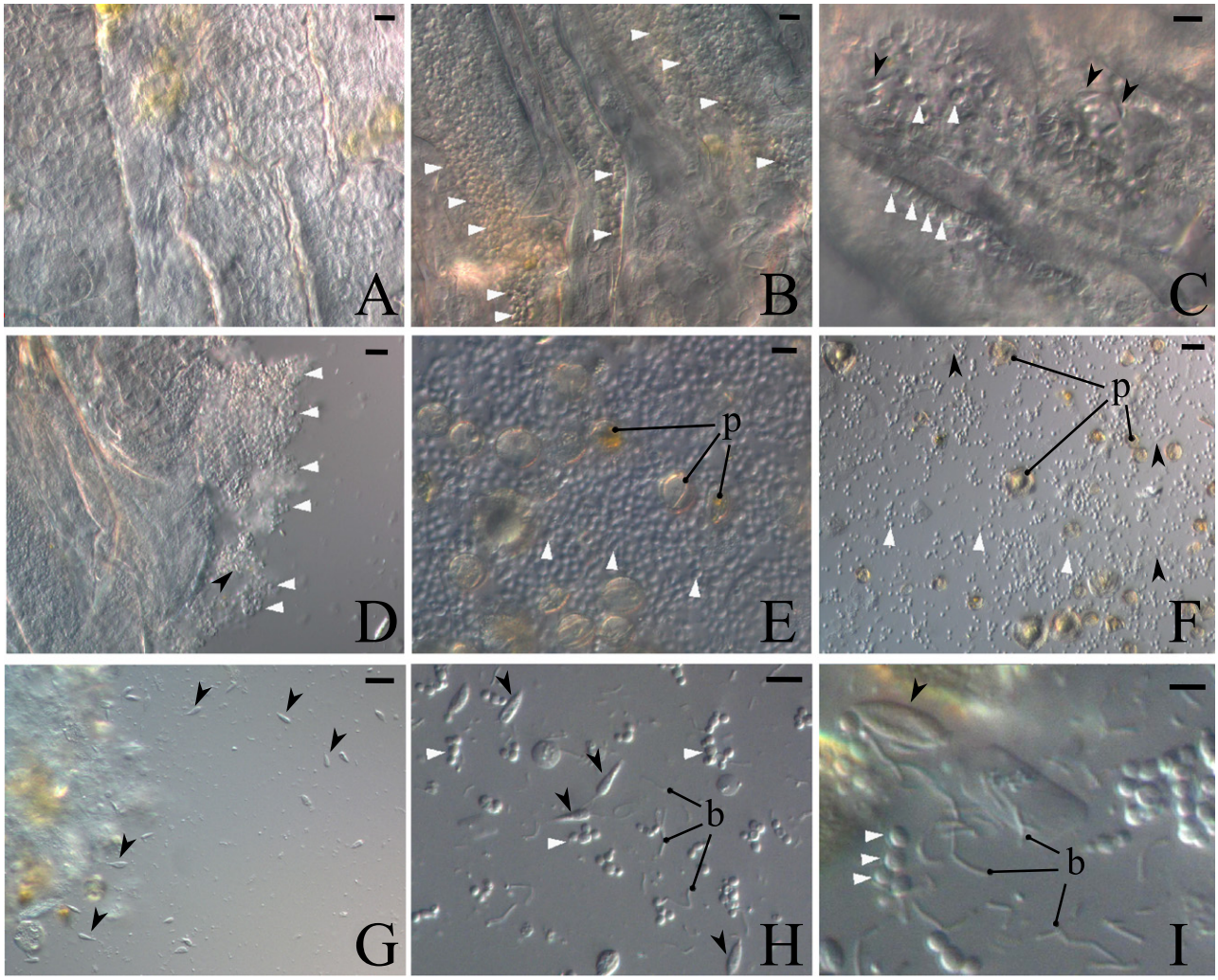

Figure 6 Light microscopy of *Lotmaria passim* n. gen., n. sp. strain BRL in vivo following experimental inoculation of *Apis mellifera* showing both spheroid (white arrowheads) and promastigote (black arrowheads) morphology. **A**. Tissue from lower ileum of uninfected control bee. **B, C**. Spheroids form a single, dense layer along the lumenal surface of the lower ileum with several promastigotes among them. **D**. Spheroid layer peeling away from the lumenal surface of the ileum where it was cut just anterior to the rectum. **E, F**. Rectal tissue macerates with typical dense colonization by spheroids among pollen grains ("p") and several promastigotes (F). **G–I**. Free swimming promastigotes from dissected hindgut tissues among dislodged spheroids and unidentified bacteria ("b") from the rectum (H, I). Promastigotes showed identical morphology to those from axenic culture, including the broad, deep lateral groove (I). Scale bars: 10 μm (B, C, E, H–I), 20 μm (A, D, F, G).

of observation, apparently having cleared the infection. Neither scarring nor specific colonization of the pylorus as described by Lotmar (1946) was observed from any of these experimental inoculations. None of the sugar water control group bees had visually detectable trypanosomatids (n = 20).

DISCUSSION

This study was spurred by a recognized need to contrast recent trypanosomatid isolates from honey bees to type strain species of *C. mellificae* (Evans and Schwarz 2011), with building evidence provided by a study that contrasted healthy vs. diseased colonies and identified trypanosomatid sequences as highly divergent from *C. mellificae* strain 30254 (Cornman et al. 2012). This study further fueled interest with the finding that these were the most prevalent nonviral parasites in honey bee colonies. The establishment of two recent honey bee Trypanosomatidae strains in axenic culture, SF (Runckel et al. 2011) and BRL (this study), allowed us to reliably obtain genetic data and contrast it with the putative axenic type strains of *C. mellificae*. Our analyses of *gGAPDH*, *SSU*, *Cytb*, and *ITS1-5.8S-ITS2* loci from these four strains consistently resolved stains BRL/SF and strains 30254/30862 into two nonoverlapping taxonomic groups, both of which were nested within the Leishmaniinae subfamily. Trypanosomatidae strains BRL and SF represent a novel taxonomic unit

distinct from any previously published characterizations and Trypanosomatidae strains 30254 and 30862 are conspecifics of the previously designated taxon *C. mellificae*. For these reasons, strains BRL and SF are denominated and described as type specimens for a novel taxon, *Lotmaria passim* n. gen., n. sp. in accordance with ICZN guidelines (ICZN 1999).

Phylogenetics using *gGAPDH-SSU* placed all Hymenoptera trypanosomatids within a novel clade (Clade 3) of the Leishmaniinae subfamily that includes the following lineages: (1) *L. passim* from *A. mellifera* (including strains BRL/haplotype B, SF/haplotype A), (2) *L. passim* from *A. cerana* (China haplotype C), (3) *C. mellificae* from *A. mellifera* (strain 30254) (4) *C. mellificae* from *V. squamosa* (strain 30862) (5) *C. bombi* from *Bombus lucorum* and (6) *C. expoeki* from *B. lucorum*. Importantly, this shows that these species shared a common ancestor that was different from the common ancestor of species within the *Leishmania* clade (Clade 1) and the *Crithidia* clade (Clade 2). The phylogenetic placement of hymenopteran trypanosomatids to the same clade within the Leishmaniinae, referred to here as Clade 3, has been a consistent finding (Morimoto et al. 2012; Runckel et al. 2014; Schmid-Hempel and Tognazzo 2010). In addition to the hymenopteran trypanosomatids, we show that Clade 3 also contains species from Hemiptera hosts (*Crithidia abscondita*, *Leptomonas jaderae*, *Leptomonas neopamerae*, *Leptomonas podlipaevi*, *Leptomonas pyrrhocoris*, *Leptomonas scantii*, and *Leptomonas seymouri*). Review of studies that have included only the hemipteran trypanosomatids from Clade 3 confirm the common ancestry of these seven species (e.g. Kostygov et al. 2014; Yurchenko et al. 2014) and the validity of Clade 3 as a distinct lineage of this subfamily. Single gene analyses of *gGAPDH* sequences corroborated this result, but *SSU* data alone did not resolve any phylogenetic positions of taxa within the Leishmaniinae. Few *Cytb* accessions are currently available at present to decipher broader relationships among the Leishmaniinae taxa, yet we found this to be the most valuable locus tested to genetically differentiate honey bee and bumblebee Trypanosomatidae strains from one another, as found in previous work (Morimoto et al. 2012; Schmid-Hempel and Tognazzo 2010). These findings confirm the value of kDNA for resolving strain differences within monoxenous trypanosomatids (Wallace et al. 1983), similar to what has been found in dixenous species (e.g. Asato et al. 2009; Spotorno et al. 2008). We note that the production of mature *Cytb* mRNAs in trypanosomatids may include the addition or, more rarely, the deletion of uridine monophosphate(s) and thus the sequences may differ from the pre-edited genomic DNA (Feagin et al. 1988), which could introduce error to phylogenetic analysis. Sequence diversity summarized in Table 1 was obtained only from genomic DNA. All *Cytb* sequences analyzed in Fig. 2 are pre-edited except the following mRNA-derived sequence fragments: Belgium (*A. mellifera*) 151-p3(×19), HapA Japan (*A. mellifera*) AB716358.1, HapB Japan (*A. mellifera*) AB716359.1 and HapC China (*Apis cerana*) AB744129.1. All mRNA-derived sequence fragments except HapC (AB744129.1) were compared to pre-edited sequences of homologous strains and showed no evidence of mRNA editing across the region of the gene investigated, which was located at the mid-region of the gene (approximately corresponding to positions 177–592 of full-length pre-edited *Cytb* from *L. braziliensis* AB434681.1). These findings are consistent with current understanding that RNA editing only occurs at the 5′ termini of *Cytb* transcripts in the Trypanosomatidae family (reviewed in Speijer 2008).

Trypanosomatid systematics originally used morphological features to define monoxenous taxa to genera, but it soon became apparent that this was creating immense confusion since many were indistinguishable due to intraspecific polymorphism and homoplasies across lineages. This was particularly true for taxa assigned to the genus *Leptomonas* or *Crithidia* based on two morphotypic homoplasies: promastigotes and choanomastigotes, respectively. As a first attempt to reduce the confusion and refine trypanosomatid taxonomy, recommendations were put forth for new species descriptions to use not only host type and cell morphology but also the establishment of axenic cell cultures from which kDNA fingerprinting, nutritional requirements, and growth parameters could be assessed (Wallace et al. 1983). Although most of these recommendations were rarely applied, establishment of axenic cell cultures did gain support and resulted in an invaluable resource of monoxenous trypanosomatid cell lines that were archived, primarily at ATCC. Because of such archived specimens (ATCC 30254 and ATCC 30862), we were able to determine the honey bee trypanosomatid *C. mellificae* is distinct from the novel honey bee isolates we define here as *L. passim*.

Current standards for protist descriptions and taxonomy require genetic data, which by default, becomes the sole determinant for taxa that cannot be reliably distinguished from other genera and/or species by morphological features. Among the Trypanosomatidae, the current broadly standard loci are *gGAPDH* and *SSU*, with *Cytb* a standard locus for some taxa. Phylogenetics using primarily *gGAPDH* and *SSU* either individually or concatenated have confirmed that classical taxonomic assignments based on morphology do not accurately reflect the evolutionary history of some lineages. For a time, *Crithidia* and *Leptomonas* were accepted as polyphyletic but it is now easy to explain that this is a result of traditional genus assignment misled by morphotypic homoplasies, which probably reflect convergent evolution rather than evolution from a common ancestor. Despite this recognition and self admonishment for doing so, authors have continued to assign new Trypanosomatidae taxa based on cell morphology to the limited, classically defined genera that are deeply entrenched in the literature instead of defining novel genera in accordance with the current standards of phylogenetics. The massive undertaking of restructuring monoxenous trypanosomtid taxa based on phylogenetics is underway, by validation and new assignments to existing genera (Borghesan et al. 2013; Teixeira et al. 2011) and by erecting novel genera (Kostygov et al. 2014; Votýpka et al. 2013, 2014), to which our report contributes.

Novel genus assignment of strains BRL and SF

As a member of the novel Clade 3, strains BRL and SF are shown to be evolutionarily associated with other species classically assigned to *Crithidia* and *Leptomonas* based on morphology. *Crithidia fasciculata* Léger, 1904 is the type species for the genus and is part of a distinct clade ("Crithidia" Clade 2) within Leishmaniinae shown here and similarly in prior work (e.g. "C" clade in Yurchenko et al. 2009; "Cf" clade in Jirků et al. 2012). Since phylogenetics did not place strains BRL and SF within the accepted type species clade, we could not justify assigning it to this genus.

Validity of the genus *Leptomonas* Kent 1880 was brought into question at its inception by Kent himself (Kent 1880) when he described the type species *Leptomonas bütschlii* Kent 1880 from an aquatic nematode (Nematoda; Adenophorea; Enoplida; Tobrilidae; *Tobrilus gracilis* [syn. *Trilobus gracilis*]). Nonetheless, a parasitic flagellate discovered in the hindgut of a water scorpion (Hemiptera, Nepidae, *Nepa*) shared the same general cell features described from the *Leptomonas* type species and was assigned to this genus as *Leptomonas jaculum* Léger, 1902. This paved the way for subsequent genus denominations of insect flagellates with a promastigote morphotype, the validity of which has been in question for decades (Maslov et al. 2013; Wallace 1966). Although no type material for *L. bütschlii* or *L. jaculum* are available to our knowledge, a trypanosomatid was recently isolated from the same host genus from which *L. jaculum* was originally described (*Nepa* sp.) and established as a "neotype" (Kostygov and Frolov 2007). If this is accepted, it represents the type material for the first insect Trypanosomatidae assigned to the genus *Leptomonas*. Phylogenetics using type DNA from this neotype (EF184218) and other Trypanosomatidae sequences established the phylogenetic clade for *L. jaculum* (Clade 5 in Maslov et al. 2013), which is not a member of the Leishmaniinae subfamily and is clearly a sister clade to the *Blastocrithidia* as shown repeatedly in numerous studies (e.g. Maslov et al. 2010; Votýpka et al. 2012; Yurchenko et al. 2009). This invalidates *Leptomonas* as a candidate genus for taxa within the Leishmaniiane subfamily. Since genus assignment of strains BRL and SF within our Clade 3 to *Crithidia* or *Leptomonas* could not be justified, we were required to denominate the new genus *Lotmaria* Evans and Schwarz, 2014.

Comparison of *L. passim* n. gen., n. sp. to prior descriptions of honey bee trypanosomatids

The characterization of a honey bee trypanosomatid by R. Lotmar (1946) provided thorough written and diagrammatic descriptions for the species proposed as *L. apis*, however, no archived type specimens nor genetic characterizations were made. Although we now know morphology cannot be the determining factor for putative species identification, it is valuable and required to address this point since it is the only information with which to make comparisons to historical characterizations. The description of *L. apis* is distinct from the BRL/SF *L. passim* taxon we propose here in two important ways. First, several careful drawings and descriptions of *L. apis* characterized the flagellated stages as having a narrow anterior end from which the flagellum originated with the cell widening distally to culminate in a large, rounded posterior end that typified this species. This morphology is more consistent with *C. mellificae* than with *L. passim*. No promastigotes with acute posteriors like those characteristic of *L. passim* were described for *L. apis*. Interestingly, a later report on trypanosomatids found in honey bees (Lom 1962) described flagellated stages as having "...the posterior end tapering and often drawn out into a sharp point", which is consistent with *L. passim* and as we show is a feature helpful to differentiate *L. passim* from *C. mellificae*. However, these trypanosomatids were attributed by the author to the genus *Crithidia* and presumed to be opportunistic infections, thus no species designation was proposed. Rarely thin, elongated forms of *L. apis* were also observed by Lotmar, which is a morphology reported from other insect trypanosomatids (e.g. *Blechomonas maslovi*, *Blechomonas wendygibsoni*, *Leptomonas spiculata*) but never for *C. mellificae* (Langridge 1966; Langridge and McGhee 1967, this report) and we have not observed such morphotypes from *L. passim*.

A second distinction we identified from Lotmar's characterization of *L. apis* was her careful description that *L. apis* specifically settled on and colonized the epithelium of the pyloric region of the gut, occasionally slightly anterior or posterior into the midgut and upper ileum. She occasionally observed flagellated stages throughout various regions of the gut, but never noted a case of colonized rectum tissue, which we show appears to be the preferred niche colonized by *L. passim* and has previously been shown as the preferred niche for *C. mellificae* as well (Langridge and McGhee 1967). These key distinctions support to the best possible ability that *L. passim*, represented by strains BRL and SF, is not the proposed *L. apis* taxon Lotmar observed in honey bees from several regions of Europe. The description of *L. apis* also does not fully support the features of *C. mellificae* as understood by Langridge and McGhee (1967) who noted Lotmar's report of *L. apis*, but did not contrast their novel species description of *C. mellificae* to *L. apis*.

We also note that the spheroid morphotype we describe for *L. passim* (nonmotile, round adherent cell) is identical to descriptions and images of *C. mellificae* in honey bees as "large numbers of the rounded parasites attached to the wall of the rectum..." (Langridge and McGhee 1967). Although these cells have the same morphology as amastigotes described from *Leishmania* spp., this term is associated with the intracellular stage within mammalian hosts. Spheroids are a diagnostic feature of both *C. mellificae* and *L. passim* (Clade 3) in honey bee hosts, but given that limited experimental in vivo work has been performed with monoxenous insect trypanosomatids, it remains to be seen if and how prevalent this morphotype is in other clades.

Identifying trypanosomatids from Hymenoptera and host specificity

Our data support that *L. passim* is currently more prevalent in honey bees than *C. mellificae*, is globally distributed, and has been present in *A. mellifera* since at least 2010 based on the earliest accessioned sequence we were able to confirm (GU321196). The majority of original trypanosomatid sequences amplified from *A. mellifera* in Belgium belonged to *L. passim* except for two *SSU* sequences from *C. mellificae*. Thus, current populations of *A. mellifera* host both species but *L. passim* predominates. It is unclear at this time why *C. mellificae* has been infrequently detected in *A. mellifera* recently, yet must have been relatively prevalent nearly 50 yr ago when it was repeatedly isolated and described in Australia (Langridge 1966; Langridge and McGhee 1967) and from the USA (type specimen accessions). We have determined that all previously accessioned sequence data designated as *C. mellificae* are incorrectly attributed and actually belong to the new species *L. passim*. Given that (1) both axenic isolates (BRL and SF) of *L. passim* were established from the USA between 2010 and 2012, (2) the vast majority of original sequence data obtained from *A. mellifera* in Belgium are from *L. passim*, and (3) all previously accessioned data from honey bees in China, Japan, Switzerland and the USA are actually lineages of *L. passim* and not *C. mellificae* as previously presumed, we conclude that *L. passim* is global and commonly infects honey bees (*A. mellifera*) while *C. mellificae* is currently comparatively infrequent. Furthermore, *L. passim* is not strictly host-specific as sequence data obtained from *A. cerana* (AB744129) also belongs to this new species.

As trypanosomatids progress through different stages of their cell cycle, variation in promastigote morphologies are well documented from *Leishmania* (Clark 1959; Wheeler et al. 2011). The flagellated stage of *C. mellificae* and *L. passim* as we have shown here can be generally useful to help discern these two species within honey bees, however, putative species designation requires genetic confirmation. Previously published light microscopy images of *C. bombi* and *C. expoeki* from bumblebees are indistinguishable from the images of *C. mellificae*, we present here and cross-infections of bumblebee and honey bee eukaryotic endoparasites do occur (Plischuk et al. 2011; Ruiz-Gonzalez and Brown 2006). Furthermore, cryptic species may be present that cannot be distinguished from one another by cell morphology, further supporting the need for genetic confirmation.

Our analyses clarify that *C. mellificae* promiscuously utilizes a wide variety of hymenopteran hosts, including not only *A. mellifera* but also *V. squamosa*, *O. bicornis* (shown here) and *Osmia cornuta* (unpublished results) and provides a contradictory example to the paradigm that insect trypanosomatids are host-specific. By contrast, *L. passim* has so far only been isolated from *A. mellifera* and in one instance *A. cerana*, supportive of the paradigm that trypanosomatids have limits to the range of hosts they can infect. Certainly, behavioral and metabolic differences may provide barriers that drive evolutionary divergence and host specificity. Previous discussions have speculated that parasites (Evans and Schwarz 2011) and trypanosomatids specifically (McGhee and Cosgrove 1980) may be delimited among honey bees due to unique social behaviors such as adult-to-larva food provision (royal jelly) and adult-to-adult food sharing (trophallaxis). Metabolic limits to cross-species susceptibility have been empirically demonstrated specifically in *A. mellifera* via challenges with trypanosomatids isolated from flies (Diptera), including *Crithidia luciliae*, *C. fasciculata*, *Crithidia culicidarum*, and *Strigomonas* (*Crithidia*) *oncopelti*, none of which established successful infections within *A. mellifera* (Lom 1962). Now, this improved characterization of inter- and intragenetic diversity among *C. mellificae* and *L. passim* provides the foundation for future work on host diversity and distribution that will elaborate on the life history of these key representatives of monoxenous trypanosomatids.

TAXONOMIC SUMMARY

Super-group. Excavata (Cavalier-Smith, 2002) emend. Simpson, 2003
Phylum. Euglenozoa (Cavalier-Smith, 1981) emend. Simpson, 1997
Class. Kinetoplastea Honigberg, 1963
Subclass. Metakinetoplastina Vickerman, 2004
Order. Trypanosomatida (Kent 1880) emend. Vickerman, 2004
Family. Trypanosomatidae Doflein, 1901
Subfamily. Leishmaniinae Maslov and Lukeš, 2012

Lotmaria n. gen. Evans and Schwarz, 2014

Diagnosis. Monoxenous parasites strictly of the Leishmaniinae subfamily and part of a distinct phylogenetic clade, referred to here as Clade 3, that currently includes taxa assigned to two other genera based on classical morphology, which is now recognized as unacceptable for taxonomic characterization: *C. abscondita*, *C. bombi*, *C. expoeki*, *C. mellificae*, *L. jaderae*, *L. neopamerae*, *L. podlipaevi*, *L. pyrrhocoris*, *L. scantii*, and *L. seymouri*.
Remarks. Phylogenetics invalidate *Leptomonas* Kent 1880 as a genus assignment for taxa within the Leishmaniianae subfamily. Phylogenetics also show that species assigned to the genus *Crithidia* Léger, 1904 must fall within the lineage associated with the type species for this genus, *C. fasciculata*, referred to here as Clade 2.
Type species. *Lotmaria passim* n. sp.
Etymology. The genus is named in honor of microbiologist and honey bee expert Ruth Lotmar, who produced extremely detailed reports of trypanosomatids from Hymenoptera in the middle of the 20th century. Ruth Lotmar was chosen as a representative for the many Hymenoptera trypanosomatid researchers whose combined work has pioneered an understanding of the host-parasite biology of monoxenous insect trypanosomes and to underscore the value Hymenoptera trypanosomatids have made

toward improved understanding of Trypanosomatidae evolutionary history. The genus suffix "-ia" was chosen to show phylogenetic affiliation with the two other prominent clades of the Leishmaniinae subfamily, *Leishmania* and *Crithidia*.

Lotmaria passim n. sp. Schwarz, 2014

Diagnosis. Promastigotes are lanceolate to tear-drop shaped, have a single free flagellum lacking a membrane that inserts into a flagellar pocket opening at the broad, rounded anterior end of the cell. Length 7.44 μm (4.66–11.40 μm), width 3.15 μm (1.50–4.65 μm). Promastigotes are horizontally compressed with a deep groove oblique to the anterior–posterior axis, creating a wide axis and a narrow axis tapering to a typically caudate posterior extension. The kinetoplast is anterior to the nucleus. A short spicule (finger-like projection) often occurs at the opening of the flagellar pocket adjacent to the flagellum, visible only using electron microscopy. Spheroids (diam. = 3–4 μm) adhere to the gut wall in a single layer and often in dense aggregates, particularly among the rectal papillae (anterior rectum) and into the lower ileum. Polymorphic cell stages that range between the described promastigote to the spheroid form may be seen.

Type taxon. Hapantotype strain BRL (ATCC PRA-422).

Type host. In hind gut (ileum) of adult female *A. mellifera ligustica* (Hymenoptera, Apidae).

Other hosts. *Apis cerana*.

Type locality. U.S. Department of Agriculture Bee Research Lab apiary in Beltsville, Maryland, USA (39°2′26.09″N, 76°51′42.25″W).

Type material. Giemsa-stained hapantotype specimen mounts have been deposited to the U.S. National Parasite Collection (USNPC) in Beltsville, Maryland, USA for *L. passim* n. gen., n. sp. strain BRL (USNPC no. 108271.00) as well as parahapantotype mounts of *L. passim* n. gen., n. sp. strain SF (USNPC no. 108272.00). Cultures of the hapantotype strain BRL have been accessioned and are available (ATCC PRA-422, Protistology Collection in Manassas, Virginia, VA, USA).

Etymology. The species name is derived from the Latin word *passim* meaning "everywhere" in reference to its global and pervasive distribution within honey bees. The species name also contains the anagram "apis m" for its type host species *A. mellifera*.

Gene sequences. The species is identified by the following unique DNA sequences obtained from the *L. passim* n. gen., n. sp. hapantotype strain BRL and deposited to GenBank™: *gGAPDH* (KJ713353 to KJ713356), *SSU rRNA* (KJ713377 and KJ713378), *Cytb* (KJ684960 to KJ684964), and *ITS1*, *5.8S*, and *ITS2* region (KJ722737 to KJ722744). Unique genetic sequences from the *L. passim* n. gen., n. sp. parahapantotype strain SF deposited to GenBank™ may also be used: *gGAPDH* (KJ713346 to KJ713352), *SSU rRNA* (KJ713371 and KJ713376), *Cytb* (KJ684955 to KJ684959), and *ITS1*, *5.8S*, and *ITS2* region (KJ722728 to KJ722736).

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LITERATURE CITED

Adl, S. M., Simpson, A. G. B., Farmer, M. A., Andersen, R. A., Anderson, O. R., Barta, J. R., Bowser, S. S., Brugerolle, G., Fensome, R. A., Fredericq, S., James, T. Y., Karpov, S., Kugrens, P., Krug, J., Lane, C. E., Lewis, L. A., Lodge, J., Lynn, D. H., Mann, D. G., McCourt, R. M., Mendoza, L., Moestrup, Ø., Mozley-Standridge, S. E., Nerad, T. A., Shearer, C. A., Smirnov, A. V., Spiegel, F. W. & Taylor, M. F. J. R. 2005. The new higher level classification of eukaryotes with emphasis on the taxonomy of protists. *J. Eukaryot. Microbiol.*, 52:399–451.

Adl, S. M., Simpson, A. G. B., Lane, C. E., Lukeš, J., Bass, D., Bowser, S. S., Brown, M. W., Burki, F., Dunthorn, M., Hampl, V., Heiss, A., Hoppenrath, M., Lara, E., le Gall, L., Lynn, D. H., McManus, H., Mitchell, E. A. D., Mozley-Standridge, S. E., Parfrey, L. W., Pawlowski, J., Rueckert, S., Shadwick, L., Schoch, C. L., Smirnov, A. V. & Spiegel, F. W. 2012. The revised classification of eukaryotes. *J. Eukaryot. Microbiol.*, 59:429–493.

Asato, Y., Oshiro, M., Myint, C. K., Yamamoto, Y., Kato, H., Marco, J. D., Mimori, T., Gomez, E. A. L., Hashiguchi, Y. & Uezato, H. 2009. Phylogenetic analysis of the genus *Leishmania* by cytochrome b gene sequencing. *Exp. Parasitol.*, 121:352–361.

Borghesan, T. C., Ferreira, R. C., Takata, C. S. A., Campaner, M., Borda, C. C., Paiva, F., Milder, R. V., Teixeira, M. M. G. & Camargo, E. P. 2013. Molecular phylogenetic redefinition of *Herpetomonas* (Kinetoplastea, Trypanosomatidae), a genus of insect parasites associated with flies. *Protist*, 164:129–152.

Clark, T. B. 1959. Comparative morphology of four genera of Trypanosomatidae. *J. Protozool.*, 6:227–232.

Cornman, R. S., Tarpy, D. R., Chen, Y., Jeffreys, L., Lopez, D., Pettis, J. S., van Engelsdorp, D. & Evans, J. D. 2012. Pathogen webs in collapsing honey bee colonies. *PLoS ONE*, 7:e43562.

Cox-Foster, D. L., Conlan, S., Holmes, E. C., Palacios, G., Evand, J. D., Moran, N. A., Quan, P.-L., Briese, T., Hornig, M., Geiser, D. M., Martinson, V., van Engelsdorp, D., Kalkstein, A. L., Drysdale, A., Hui, J., Zhai, J., Cui, L., Hutchison, S. K., Simons, J. F., Egholm, M., Pettis, J. S. & Lipkin, W. I. 2007. A metagenomic survey of microbes in honey bee colony collapse disorder. *Science*, 318:283–287.

Dietemann, V., Ellis, J. D. & Neumann, P. 2013. COLOSS BEEBOOK: Standard Methods for *Apis mellifera* Research. International Bee Research Association, Treforest. p. 1–614.

Dwyer, D. M., Langreth, S. G. & Dwyer, N. K. 1974. Evidence for a polysaccharide surface coat in the developmental stages of *Leishmania donovani*: a fine structure-cytochemical study. *Z. Parasitenk.*, 43:227–249.

Edgar, R. C. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.*, 32:1792–1797.

van Engelsdorp, D., Evans, J. D., Saegerman, C., Mullin, C., Haubruge, E., Nguyen, B. K., Frazier, M., Frazier, J., Cox-Foster, D., Chen, Y., Underwood, R., Tarpy, D. R. & Pettis, J. S. 2009. Colony collapse disorder: a descriptive study. *PLoS ONE*, 4:e6481.

Evans, J. D. & Schwarz, R. S. 2011. Bees brought to their knees: microbes affecting honey bee health. *Trends Microbiol.*, 19:614–620.

Fanthan, H. B. & Porter, A. 1912. Note on certain protozoa found in bees. *Suppl. J. Board Agricul.*, 19:138.

Feagin, J. E., Shaw, J. M., Simpson, L. & Stuart, K. 1988. Creation of AUG initiation codons by addition of uridines within cytochrome b transcripts of kinetoplastids. *Proc. Natl Acad. Sci. USA*, 85:539–543.

Forsgren, E. & Fries, I. 2010. Comparative virulence of *Nosema ceranae* and *Nosema apis* in individual European honey bees. *Vet. Parasitol.*, 170:212–217.

Fyg, W. 1954. Über das Vorkommen von Flagellaten im Rectum der Honigbiene (*Apis mellifica* L.). *Mitteil. Schweiz. Entomol. Gesell.*, 27:423–428.

Genersch, E., Ashiralieva, A. & Fries, I. 2005. Strain- and genotype-specific differences in virulence of *Paenibacillus larvae* subsp. *larvae*, a bacterial pathogen causing american foulbrood disease in honeybees. *Appl. Env. Microbiol.*, 71:7551–7555.

Higes, M., Meana, A., Bartolomé, C., Botías, C. & Martín-Hernández, R. 2013. *Nosema ceranae* (Microsoporidia), a controversial 21st century honey bee pathogen. *Env. Microbiol. Rep.*, 5:17–29.

Hoare, C. A. & Wallace, F. G. 1966. Developmental stages of trypanosomatid flagellates: a new terminology. *Nature*, 212:1385–1386.

ICZN 1999. International Code of Zoological Nomenclature, 4th ed. The International Trust for Zoological Nomenclature, London.

Jirků, M., Yurchenko, V. Y., Lukeš, J. & Maslov, D. A. 2012. New species of insect trypanosomatids from Costa Rica and the proposal for a new subfamily within the Trypanosomatidae. *J. Eukaryot. Microbiol.*, 59:537–547.

Kent, W. S. 1880. A Manual of the Infusoria, Vol. 1. David Bogue, London. p. 1–472.

Klee, J., Besana, A. M., Genersch, E., Gisder, S., Nanetti, A., Tam, D. Q., Chinh, T. X., Puerta, F., Ruz, J. M., Kryger, P., Message, D., Hatjina, F., Korpela, S., Fries, I. & Paxton, R. J. 2007. Widespread dispersal of the microsporidian *Nosema ceranae*, an emergent pathogen of the western honey bee, *Apis mellifera*. *J. Invert. Pathol.*, 96:1–10.

Kostygov, A. Y. & Frolov, A. 2007. *Leptomonas jaculum* (Leger, 1902) Woodcock 1914: a leptomonas or a blastocrithidia? *Parazitologiia*, 41:126–136.

Kostygov, A. Y., Grybchuk-Ieremenko, A., Malysheva, M. N., Frolov, A. O. & Yurchenko, V. 2014. Molecular revision of the genus *Wallaceina*. *Protist*, 165:594–604.

Langridge, D. F. 1966. Flagellated protozoa (Trypanosomidae) in the honey bee, *Apis mellifera*, in Australia. *J. Invert. Pathol.*, 8:124–126.

Langridge, D. F. & McGhee, R. B. 1967. *Crithidia mellificae* n. sp. an acidophilic trypanosomatid of the honey bee *Apis mellifera*. *J. Protozool.*, 14:485–487.

Lom, J. 1962. The occurence of a *Crithidia*-species within the gut of the honey-bee, *Apis mellifica* L. *Entom. Mém. S.*, 2:91–93.

Lotmar, R. 1946. Üuber flagellaten und bakterien im düundarm der honigbiene (*Apis mellifica*). *Beih. Schweiz. Bienen-Zeit.*, 2:49–76.

Macarisin, D., Bauchan, G. & Fayer, R. 2010. *Spinacia oleracea* L. leaf stomata harboring *Cryptosporidium parvum* oocysts: a potential threat to food safety. *Appl. Env. Microbiol.*, 76:555–559.

Macarisin, D., O'Brien, C., Fayer, R., Bauchan, G. & Jenkins, M. 2012. Immunolocalization of β- and δ-giardin within the ventral disk in trophozoites of *Giardia duodenalis* using multiplex laser scanning confocal microscopy. *Parasitol. Res.*, 111:241–248.

Maslov, D. A., Votypka, J., Yurchenko, V. & Lukeš, J. 2013. Diversity and phylogeny of insect trypanosomatids: all that is hidden shall be revealed. *Trends Parasitol.*, 29:43–52.

Maslov, D. A., Yurchenko, V. Y., Jirků, M. & Lukeš, J. 2010. Two new species of trypanosomatid parasites isolated from Heteroptera in Costa Rica. *J. Eukaryot. Microbiol.*, 57:177–188.

McGhee, R. B. & Cosgrove, W. B. 1980. Biology and physiology of the lower Trypanosomatidae. *Microbiol. Rev.*, 44:140–173.

Merzlyak, E., Yurchenko, V., Kolesnikov, A. A., Alexandrov, K., Podlipaev, S. A. & Maslov, D. A. 2001. Diversity and phylogeny of insect trypanosomatids based on small subunit rRNA genes: polyphyly of *Leptomonas* and *Blastocrithidia*. *J. Eukaryot. Microbiol.*, 48:161–169.

Moran, N. A., Hansen, A. K., Powell, J. E. & Sabree, Z. L. 2012. Distinctive gut microbiota of honey bees assessed using deep sampling from individual worker bees. *PLoS ONE*, 7(4):e36393.

Morimoto, T., Kojima, Y., Yoshiyama, M., Kimura, K., Yang, B., Peng, G. & Kadowaki, T. 2012. Molecular detection of protozoan parasites infecting Apis mellifera colonies in Japan. *Environ. Microbiol. Rep.*, 5:74–77.

Orantes-Bermejo, F. J. 1999. A scientific note on the prevalence of trypanosomatid parasites of honey bees (*Apis mellifera* L.) in southern Spain. *Apidologie*, 30:77–78.

Plischuk, S., Meeus, I., Smagghe, G. & Lange, C. E. 2011. *Apicystis bombi* (Apicomplexa: Neogregarinorida) parasitizing *Apis mellifera* and *Bombus terrestris* (Hympenoptera: Apidae) in Argentina. *Environ. Microbiol. Rep.*, 3:565–568.

Popp, M. & Latorff, H. M. G. 2011. A quantitative in vitro cultivation technique to determine cell number and growth rates in strains of *Crithidia bombi* (Trypanosomatidae), a parasite of bumblebees. *J. Eukaryot. Microbiol.*, 58:7–10.

Ravoet, J., Maharramov, J., Meeus, I., de Smet, L., Wenseleers, T., Smagghe, G. & de Graaf, D. C. 2013. Comprehensive bee pathogen screening in Belgium reveals *Crithidia mellificae* as a new contributory factor to winter mortality. *PLoS ONE*, 8: e72443.

Ruiz-Gonzalez, M. X. & Brown, M. J. F. 2006. Honey bee and bumblebee trypanosomatids: specificity and potential for transmission. *Ecol. Entomol.*, 31:616–622.

Runckel, C., DeRisi, J. & Flenniken, M. L. 2014. A draft genome of the honey bee trypanosomatid parasite *Crithidia mellificae*. *PLoS ONE*, 9:e95057.

Runckel, C., Flenniken, M. L., Engel, J. C., Ruby, J. G., Ganem, D., Andino, R. & DeRisi, J. L. 2011. Temporal analysis of the honey bee microbiome reveals four novel viruses and seasonal prevalence of known viruses, *Nosema*, and *Crithidia*. *PLoS ONE*, 6:e20656.

Schmid-Hempel, R. & Tognazzo, M. 2010. Molecular divergence defines two distinct lineages of *Crithidia bombi* (Trypanosomati-

dae), parasites of bumblebees. *J. Eukaryot. Microbiol.*, 57:337–345.
Schwarz, R. S. & Evans, J. D. 2013. Single and mixed-species trypanosome and microsporidia infections elicit distinct, ephemeral cellular and humoral immune responses in honey bees. *Dev. Comp. Immunol.*, 40:300–310.
Speijer, D. 2008. Evolutionary aspects of RNA editing. *In*: Göringer, H. U. (ed.), RNA Editing. Springer-Verlag, Berlin Heidelberg. p. 199–227.
Spotorno, A. E., Córdova, L. & Solari, A. 2008. Differentiation of *Trypanosoma cruzi* I subgroups through characterization of cytochrome b gene sequences. *Inf. Gen. Evol.*, 8: 898–900.
Stöver, B. C. & Müller, K. F. 2010. TreeGraph 2: combining and visualizing evidence from different phylogenetic analyses. *BMC Bioinformatics*, 11:7.
Tamura, K., Stecher, G., Peterson, D., Filipski, A. & Kumar, S. 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Mol. Biol. Evol.*, 30:2725–2729.
Teixeira, M. M. G., Borghesan, T. C., Ferreira, R. C., Santos, M. A., Takata, C. S. A., Campaner, M., Nunes, V. L. B., Milder, R. V., de Souza, W. & Camargo, E. P. 2011. Phylogenetic validation of the genera *Angomonas* and *Strigomonas* of trypanosomatids harboring bacterial endosymbionts with the description of new species of trypanosomatids and of proteobacterial symbionts. *Protist*, 162:503–524.
Teixeira, É. W., Message, D., Chen, Y., Pettis, J. & Evans, J. D. 2008. First metagenomic analysis of microorganisms in honey bees from Brazil. *B. Indústr. Anim.*, 65:355–361.
Vásquez, A., Forsgren, E., Fries, I., Paxton, R. J., Flaberg, E., Szekely, L. & Olofsson, T. C. 2012. Symbionts as major modulators of insect health: lactic acid bacteria and honeybees. *PLoS ONE*, 7(3):e33188.
Vickerman, K. 1976. The diversity of the kinetoplastid flagellates. *In*: Lumsden, W. H. R. & Evans, D. A. (ed.), Biology of the Kinetoplastida. Academic Press, London. p. 1–34.
Vickerman, K. 1994. The evolutionary expansion fo the trypanosomatid flagellates. *Int. J. Parasitol.*, 24:1317–1331.
Votýpka, J., Klepetková, H., Jirků, M., Kment, P. & Lukeš, J. 2012. Phylogenetic relationships of trypanosomatids parasitising true bugs (Insecta: Heteroptera) in sub-Saharan Africa. *Int. J. Parasitol.*, 42:489–500.
Votýpka, J., Kostygov, A. Y., Kraeva, N., Grybchuk-Ieremenko, A., Tesarová, M., Grybchuk, D., Lukeš, J. & Yurchenko, V. 2014. *Kentomonas* gen. n., a new genus of endosymbiont-containing Trypanosomatids of Strigomonadinae subfam. n. *Protist*. 165:825–838.
Votýpka, J., Suková, E., Kraeva, N., Ishemgulova, A., Duží, I., Lukeš, J. & Yurchenko, V. 2013. Diversity of trypanosomatids (Kinetoplastea: Trypanosomatidae) parasitizing fleas (Insecta: Siphonaptera) and description of a new genus *Blechomonas* gen. n. *Protist*, 164:763–781.
Wallace, F. G. 1966. The trypanosomatid parasites of insects and arachnids. *Exp. Parasitol.*, 18:124–193.
Wallace, F. G., Camargo, E. P., McGhee, R. B. & Roitman, I. 1983. Guidelines for the description of new species of lower trypanosomatids. *J. Protozool.*, 30:308–313.
Wheeler, R. J., Gluenz, E. & Gull, K. 2011. The cell cycle of *Leishmania*: morphogenetic events and their implications for parasite biology. *Mol. Microbiol.*, 79:647–662.
Wheeler, R. J., Gluenz, E. & Gull, K. 2013. The limits on trypanosomatid morphological diversity. *PLoS ONE*, 8:e79581.
Yang, B., Peng, G., Li, T. & Kadowaki, T. 2013. Molecular and phylogenetic characterization of honey bee viruses, *Nosema* microsporidia, protozoan parasites, and parasitic mites in China. *Ecol. Evol.*, 3:298–311.
Yurchenko, V. Y., Lukeš, J., Jirků, M. & Maslov, D. A. 2009. Selective recovery of the cultivation-prone components from mixed trypanosomatid infections: a case of several novel species isolated from neotropical Heteroptera. *Int. J. Syst. Evol. Microbiol.*, 59:893–909.
Yurchenko, V., Lukeš, J., Jirků, M., Zeledón, R. & Maslov, D. A. 2006b. *Leptomonas costaricensis* sp. n. (Kinetoplastea: Trypanosomatidae), a member of the novel phylogenetic group of insect trypanosomatids closely related to the genus *Leishmania*. *Parasitology*, 133:537–546.
Yurchenko, V. Y., Lukeš, J., Tesařová, M., Jirků, M. & Maslov, D. A. 2008. Morphological discordance of the new trypanosomatid species phylogenetically associated with the genus *Crithidia*. *Protist*, 159:99–114.
Yurchenko, V., Lukeš, J., Xu, X. & Maslov, D. A. 2006a. An integrated morphological and molecular approach to a new species description in the Trypanosomatidae: the case of *Leptomonas podlipaevi* n. sp., a parasite of *Boisea rubrolineata* (Hemiptera: Rhopalidae). *J. Eukaryot. Microbiol.*, 53:103–111.
Yurchenko, V. Y., Votýpka, J., Tesařová, M., Klepetková, H., Kraeva, N., Jirků, M. & Lukeš, J. 2014. Ultrastructure and molecular phylogeny of four new species of monoxenous trypanosomatids from flies (Diptera: Brachycera) with redefinition of the genus *Wallaceina*. *Folia Parasitol.*, 61:97–112.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1. Trypanosomatid type specimen information and accession numbers from this study.
Table S2. Predicted gGAPDH and Cytb protein identity among axenic trypanosomatid cell lines.
Table S3. Unique amplicons isolated from the *gGAPDH* locus.
Table S4. Unique amplicons isolated from the *SSU* locus.
Table S5. Unique amplicons isolated from the *Cytb* locus.
Table S6. Unique amplicons isolated from the *ITS1-5.8S-ITS2* locus.
Figure S1. *gGAPDH* single gene ML phylogeny of the Leishmaniinae.
Figure S2. *SSU* single gene ML phylogeny of the Leishmaniinae.
Figure S3. *gGAPDH* single gene ML phylogeny using all Trypanosomatidae.
Figure S4. *gGAPDH* single gene NJ phylogeny of the Leishmaniinae.
Figure S5. *SSU* single gene ML phylogeny using all Trypanosomatidae.
Figure S6. *SSU* single gene NJ phylogeny using all Trypanosomatidae.
Figure S7. *Cytb* single gene ML phylogeny using all Trypanosomatidae.
Figure S8. *Cytb* single gene NJ phylogeny using all Trypanosomatidae.